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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 9/127, 38/37	A1	(11) International Publication Number: WO 99/55306 (43) International Publication Date: 4 November 1999 (04.11.99)
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(54) Title: PHARMACEUTICAL COMPOSITION COMPRISING FACTOR VIII AND NEUTRAL LIPOSOMES (57) Abstract A pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of a protein or polypeptide and substantially neutral colloidal particles. The particles comprise approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer which carries substantially no net charge. The protein or polypeptide is capable of externally binding the colloidal particles, or is capable of binding polyethylene glycol, and is not encapsulated in the colloidal particles. A preferred protein is factor VIII, whose half-life is extended and which is protected from serum inhibitor antibodies by injecting it as a component of the composition.		

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PHARMACEUTICAL COMPOSITION COMPRISING FACTOR VIII AND NEUTRAL LIPOSOMES

FIELD OF THE INVENTION

The present invention relates to a stable pharmaceutical formulation for the slow release of coagulation promoting substances for the treatment of blood coagulation disorders.

5 BACKGROUND OF THE INVENTION

Hemophilia A is one of the most frequently occurring inherited coagulation disorders. Patients with hemophilia A are prone to frequent hemorrhages as a result of one or more misfunctions of the coagulation system. One of the causes of hemophilia is a shortage of Factor VIII (FVIII)
10 in the blood. This problem can be treated with Factor VIII concentrates. However, in about 15% of the patients the occurrence results of Factor VIII neutralizing antibodies, so-called inhibitors, whereby a therapy with Factor VIII concentrates is hardly possible.

Two basic approaches have been described in the literature to
15 protect FVIII from inactivation by inhibitors.

WO/80/01456 to Hemker discloses a pharmaceutical composition suitable for oral administration comprising FVIII incorporated within liposomes of 0.5-1.0 microns formed from phospholipids. The phospholipids have a net charge, and the FVIII is incorporated between the

layers of the liposome. It is claimed that FVIII levels in the plasma remained above about 5% of the normal value for a period of 50 hours.

US 4,348,384 to Horikoshi states that a composition as described in Hemker was prepared, but did not give satisfactory results.
5 Therefore, Horikoshi incorporates a protease inhibitor into the liposome together with FVIII, in order to protect it from proteolysis. 3% of the normal plasma levels of FVIII were obtained over a period of 6 hours.

US 5,013,556 to Woodle discloses a liposome composition for use in delivering various drugs via the bloodstream. The liposome contains
10 between 1-20 mole percent of an amphipathic lipid derivatized with a polyalkylether. Here also, the drug compound is entrapped within the liposome. These liposome compositions are available commercially under the name of Stealth® vesicles (SUV's, small unilamellar vesicles comprised of phospholipid and polyethylene glycol (PEG) covalently bound to
15 phospholipid).

A further problem with this approach is that liposomes having a large diameter have a short half-life. Therefore, the liposomes must be downsized under high pressure, which can affect protein activities as in coagulation factors V and VIII.

20 In a second approach, Barrowcliffe, T.W., *et al.* (1983) J. Lab. Clin. Med. 101:34-43 teaches that mixing FVIII with phospholipid extracted from human and/or animal brain imparts significant protection to the FVIII *in vitro*. In this approach, the phospholipid is bound to the FVIII rather than encapsulating it. Kembell-Cook, G. and Barrowcliffe, T.W. (1992) Thromb.
25 Res. 67:57-71, teaches that a negatively-charged phospholipid surface is necessary for FVIII binding. Negatively charged phosphatidyl serine and phosphatidic acid were found to be highly active in binding to FVIII, while phosphatidyl choline was inactive. However, negatively-charged

phospholipids are toxic, and those derived from brain tissue may carry pathogenic agents.

EP 689,428 discloses a liposome composition comprising liposomes having an outer surface layer of hydrophilic polymer chains. A polypeptide or polysaccharide effector molecule is covalently attached to the distal ends of the polymer chains by activation of the lipid anchor prior to effector coupling.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a pharmaceutical composition comprising a protein or polypeptide for therapeutic treatment. In particular, it is an object of the present invention to provide a pharmaceutical composition comprising FVIII for the treatment of blood coagulation disorders.

It is a further object of the invention to provide FVIII in a form having an extended half-life in the bloodstream.

It is a still further object of the invention to provide a method for treating patients suffering from blood coagulation disorders, particularly hemophilia, and most particularly those having FVIII inhibitors.

In one aspect of the present invention there is provided a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, the particles comprising 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, the polymer carrying substantially no net charge, wherein the FVIII is not encapsulated in the colloidal particles.

The present invention is based on the surprising and unexpected finding that neutral phospholipids derivatized with a bio-compatible hydrophilic polymer can be used to bind FVIII and protect it from inhibitors

in the bloodstream. This provides a significant advantage over the prior art compositions, since the phospholipids used are synthetic and non-toxic, and can therefore be used *in vivo* for therapeutic treatment. Furthermore, the liposome does not encapsulate the FVIII so that smaller sized liposomes can
5 be used which have a longer half-life *in vivo*, since they are not removed by the reticuloendothelial system (RES). As will be described below in greater detail, FVIII interacts non-covalently with the polymer chains on the external surface of the liposomes, and no chemical reaction is carried out to activate the polymer chains, unlike the composition disclosed in EP 689,428.

10 In the present specification, the terms "*substantially neutral*" and "*substantially no net charge*" mean neither positively nor negatively charged. However, a very low measured charge within experimental error of zero is included within the meaning of the above terms.

The term "*therapeutically effective amount*" is to be understood
15 as referring to an amount of FVIII which results in a level of FVIII in the bloodstream having a desired therapeutic effect. Such an amount can be experimentally determined by administering compositions comprising different amounts of FVIII and measuring the level in the blood at various times after administration.

20 The amphipathic lipid used to prepare the colloidal particles is preferably a phospholipid, and may be obtained from either natural or synthetic sources. A most preferred phospholipid is phosphatidylcholine, most preferably egg-phosphatidylcholine.

The biocompatible hydrophilic polymer may include polymers
25 from the polyalkylether, polylactic or polyglycolic acid families. Preferably, the polymer is polyethylene glycol (PEG). The purpose of the polymer is to sterically stabilize the SUVs, thus preventing fusion of the vesicles *in vitro*, and allowing the vesicles to escape adsorption by the RES *in vivo*. The

polymer will preferably have a molecular weight of between about 1000 to about 5000 daltons, most preferably approximately 2000 daltons.

The colloidal particles will preferably have a mean particle diameter of between about 0.05 to about 0.4 microns, most preferably about 0.1 microns. This is to increase their circulation time *in vivo* and prevent their adsorption by the RES. The amphipathic lipid comprises approximately 1 to about 20 mole % of the particles, preferably approximately 1-5%, most preferably 5%.

A variety of known coupling reactions may be used for preparing vesicle forming lipids derivatized with hydrophilic polymers. For example, a polymer (such as PEG) may be derivatized to a lipid such as phosphatidylethanolamine (PE) through a cyanuric chloride group. Alternatively, a capped PEG may be activated with a carbonyl diimidazole coupling reagent, to form an activated imidazole compound. Other reactions are well known and are listed, e.g. in the aforementioned U.S. 5,013,556, whose contents are incorporated herein by reference.

The FVIII used in the composition of the invention is commercially available. It may be from a natural human source, or, preferably, it may be recombinantly prepared. Recombinant FVIII is commercially available, for example, Antihemophilic Factor (Recombinant), rFVIII-SQ (Pharmacia), and Kogenate, Miles Inc., Pharmaceutical Division, Elkhart, IN, U.S.A., among other suppliers.

The composition of the invention is administered parenterally, preferably iv. The prior art compositions were intended for oral use only, due to side effects caused during injection by the liposome composition. The composition of the invention, on the other hand, is not toxic by injection, apparently due to the lack of charge, among other causes. Amounts of up to 0.5gm/Kg body weight of colloidal particles according to the invention have been injected without detectable toxic symptoms. The dose is expected to be

in the approximate range of 25-75 i.u./Kg. body weight. The particle to FVIII ratio (w/unit FVIII) will preferably be between about 0.1 mg/unit and about 10 mg/unit, and most preferably, approximately 1 mg/unit. Although the free form of FVIII:C has a half-life of less than 2 hours (FVIII measured by clotting activity) in mice, FVIII administered in the composition of the invention is expected to be effective for at least 24 hours, which is the period of effective activity of the coagulation promoting compound. The composition of the invention is expected to be effective in "on demand" and prophylactic treatment of hemophilia patients, and particularly those patients who have developed FVIII inhibitor antibodies.

The effectiveness of FVIII contained in the composition of the invention may be determined by a chromogenic assay which determines FVIII activity by two consecutive steps: (1) the FVIII-dependent conversion of Factor X to Factor Xa in a coagulation-factor reagent composed of purified components, and (2) the enzymatic cleavage of a chromogenic Factor Xa substrate to yield a chromophore which can be quantified spectrophotometrically. Under appropriate assay conditions, there exists a linear relationship between the rate of Factor Xa formation and the FVIII concentration. In addition, FVIII activity may be determined by a one-stage clotting assay. This assay determines FVIII activity by the conversion of prothrombin to thrombin, which subsequently cleaves fibrinogen to form a clot composed of fibrin. FVIII activity in hemophilic mice may also be determined by measuring the survival of the mice following a tail cut.

In a further aspect of the invention, there is provided a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of a protein or polypeptide and substantially neutral colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge, wherein said

protein or polypeptide is selected from the group consisting of: (a) proteins or polypeptides capable of externally binding said colloidal particles; and (b) proteins or polypeptides capable of binding polyethylene glycol (PEG), and wherein said protein or polypeptide is not encapsulated in said colloidal particles.

The term "*proteins or polypeptides capable of externally binding said colloidal particles*" includes proteins and polypeptides which, similarly to FVIII, bind to membranes comprising phosphatidylcholine: phosphatidylserine (PC:PS) (see Haemostasis and Thrombosis. Arthur L. Bloom and Duncan P. Thomas (eds) (1987) Churchill Livingstone, pg. 179-180). Non-limiting examples of such proteins are coagulation factors such as prothrombin, Factor X and Factor V.

The term "*proteins or polypeptides capable of binding polyethylene glycol*" includes proteins and polypeptides which bind to PEG or derivatives of PEG by any non-covalent mechanism, such as ionic interactions, hydrophobic interactions, hydrogen bonds and Van der Waals attractions (Arakawa, T. and Timasheff, S.N. (1985) Biochemistry 24:6756-6762; Lee, J.C. and Lee, L.L.Y. (1981) J. Biol. Chem. 226:625-631).

BRIEF DESCRIPTION OF THE DRAWING:

In order to understand the invention and to see how it may be carried out in practice, preferred embodiments will now be described, by way of non-limiting example only, with reference to the accompanying drawing which illustrates survival of hemophilic mice injected with FVIII following a tail cut at various time periods post-injection.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

Examples 1-3

Methods and Materials

1. Egg phosphatidylcholine (E-PC) liposomes

5 A tert-butanol solution of egg phosphatidylcholine (E- PC) was prepared by dissolving 2.0 gr. E-PC, 1.9 mg α -tocopherol and fluorescein-labeled phosphatidylethanolamine (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by
10 lyophilization and the lipids reconstituted in water to 10 % w/v. The obtained liposomes were reduced in size by extruding them through a series of polycarbonate (PC) filters (0.4 μ m, 0.2 μ m, 0.1 μ m and 0.05 μ m) using the Liposofast-Basic or Liposofast-50 extruder (Avestin) to obtain liposomes of an average size of 0.1 μ m.

15

2. Egg phosphatidylcholine/polyethyleneglycol-phosphatidyl ethanolamine (E-PC/PEG-PE) liposomes

A tert-butanol solution of egg phosphatidylcholine (E- PC) and polyethyleneglycol-phosphatidyl ethanolamine (PEG-PE) was prepared by
20 mixing 0.73 gr. E-PC, 0.185 gr. PEG-PE, 0.86 mg α -tocopherol and fluorescein labeled phosphatidylethanolamine (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by lyophilization and then reconstituted in water to 10 % w/v. The obtained
25 liposomes were reduced in size as described in 1 above to obtain liposomes of an average size of 0.1 μ m.

3. Egg phosphatidylcholine-phosphatidyl glycerol (E-PC/PG) liposomes

A tert-butanol solution of egg phosphatidylcholine (E- PC) and phosphatidyl glycerol (PG) was prepared by mixing 0.822 gr. E-PC, 0.0924 gr. PG, 0.86 mg α -tocopherol and phosphatidylethanolamine fluorescein labeled (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by lyophilization and then reconstituted in water to 10 % w/v. The obtained liposomes were reduced in size as described in 1 above to obtain liposomes of an average size of 0.1 μ m.

4. Reconstitution of the human recombinant factor VIII:

Kogenate (rFVIII formulated with human albumin, Bayer) lots 70K026 and 70K027, were used in the following examples. One vial containing about 500 IU of FVIII activity was reconstituted with 2 ml water and allowed to solubilize. 200 μ l aliquots were frozen at -20°C until use.

For the preparation of albumin-depleted Kogenate, lot # 70K027 was used. 10 vials of Kogenate were reconstituted in 20 ml water and chromatographed on a hydrophilic silica gel (3-10 μ m beads). Fractions of 10ml were collected and the protein and FVIII:Ag activities were monitored.

A 50 % recovery in FVIII:Ag activity was found in one peak of fractions 4-6 and another of fractions 8-14. Since the protein assay gave a peak at fractions 9-12, fractions 4-6 were pooled, aliquoted and lyophilized for further use.

5. Hemophilic mice prepared as described in Bi, L., *et al.* (1995) Nature Genetics 10:119-121, were used.

6. FVIII:Ag activity was determined using a FVIII chromogenic assay commercially available from Dade AG, Dudinggen, Switzerland.

7. Preparation of composition and injection to hemophiliac mice
A liposomal aliquot was mixed with a predetermined volume of FVIII to obtain a FVIII:Ag activity of 5-10 IU/ml and rolled at RT to achieve
5 homogeneity.
8. Groups of 5-10 hemophiliac mice were injected IV bolus through the tail vein, with 200 or 400 μ l of the mixture. The mice were bled from the eye at regular time intervals (1h, 4hs, 8hs, 24hs, 32hs and 48hs) and
10 the FVIII:Ag activities in the plasma were followed.
9. The pharmacokinetics of FVIII was determined from the results by using the RSTRIP computation software to obtain the initial FVIII:C activity (A_0) and the half-life time ($T_{1/2}$) of the factor in the mice blood
15 circulation.

Results

1. Effect of Lipid composition on the Half-Life of Factor VIII.
Liposomes of 0.05 μ m comprising E-PC, E-PC/PEG-PE and
20 E-PC/PG were prepared, mixed with Kogenate in a 72:1 lipid to protein (w/w) ratio and injected into hemophiliac mice. As a control, Kogenate was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #1: Effect of lipid composition on the half-life of FVIII

Lipid composition	A ₀ * (IU/ml)	T _{1/2} (hs)	no. of mice
Control	2.22	4.51	18
E-PC/PEG-PE	3.20	7.84	10
E-PC	1.01	2.33	10
E-PC/PG	Not-detectable	not-detectable	10

* A₀ = initial concentration of FVIII:C

It can be seen from the table that liposomes containing E-PC /PEG-PE were the most effective since both the initial FVIII activity and the half-life time were higher for this composition than for Kogenate or Kogenate-liposome mixtures where the liposomes were composed of E-PC/PG or E-PC only.

Moreover, 40% of the mice injected with free FVIII and 100% of the mice injected with FVIII /PC complex did not exhibit any recovery of FVIII chromogenic activity, while only 10% of the mice injected with FVIII/PC+PEG exhibited the same phenomena 60 min. after injection.

2. Effect of Lipid/Protein Ratio on the Half-Life of Factor VIII.

Various lipid to protein ratios in the liposome composition were obtained by mixing various aliquots of liposomes of 0.05 μ m comprising E-PC/PEG-PE with Kogenate. These were injected into hemophiliac mice. As a control, Kogenate was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #2: Effect of lipid to protein ratio on the half-life of FVIII

lipid/prot. (w/w)	A ₀ (IU/ml)	T _{1/2} (hs)	no. of mice
134	2.26	3.3	10
32	1.61	1.91	10
5.3	3.12	1.64	10
0.89	2.69	1.5	10
Control	2.22	1.5	18

It can be seen from Table #2 that increasing the lipid/protein ratio increases the half-life time of FVIII in the blood circulation in the hemophiliac mice. The differences in the initial FVIII:C activities appear not to be related to the lipid/protein ratio.

3. Effect of different Factor VIII sources

SUVs of 0.05 μ m were prepared containing E-PC and PEG-PE (94:6 mol %), mixed with FVIII concentrates from various sources (Kogenate, Baxter and Omrixate) in a 72:1 lipid to protein ratio and injected into hemophiliac mice. As a control, each FVIII concentrate from the various sources was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #3: Effect of factor FVIII source on the half-life of FVIII

source	A ₀ (IU/ml)	T _{1/2} (hs)
Kogenate	2.22	4.51
Kogenate+ SUV's	3.36	8.60
Baxter	1.36	3.83
Baxter+ SUV's	1.08	4.45
Omrixate	2.35	3.21
Omrixate + SUV's	2.31	3.90

Mixtures containing liposomes and FVIII from Baxter or
5 Omrixate increased the half-life of the factor by 20%, when compared with
the pharmacokinetic values of the free factor, as can be seen from the above
table. The half-life of factor FVIII from Kogenate, mixed with E-PC/PEG-PE
liposomes was twice as long as compared with the free factor form.

10 **Example 4**

Methods and materials

1. Liposome preparation:

Liposomes were prepared as follows: Egg phosphatidyl choline
(EPC) and distearoyl phosphatidyl-ethanolamine methyl polyethylene glycol
15 2000 (DSPE-PEG 2000) were weighed to a ratio of 80:20 w/w (5% molar
ratio of DSPE-PEG 2000), respectively, dissolved to 10% w/v in tert-buthanol
(Reidel-de Haen), and the solution was lyophilized. The obtained dry lipid
powder was resuspended to 10% w/v in a buffer containing 130 mM NaCl, 10
mM sodium citrate, 1 mM CaCl₂ pH 7.0 to form liposomes. The liposomes

were filtered in an extruder apparatus (Avestin) through polycarbonate filters 1.2 μm , 0.2 μm and 0.1 μm in size to form liposomes of 120-140 nm in size.

2. Liposome quality control

5 Quality control of the liposomes included:

- 1) Size distribution measured by sub-micron particle analyzer (N4 plus, Coulter Electronics).
- 2) Phospholipid determination (by phosphorus).
- 3) Chemical stability of the lipids by TLC.

10 The tests were performed as described in: Barenholz, Y. and Amselem, S. (1993) in Liposome Technology, 2nd edition, Vol. I (Gregoriadis, G., ed.), CRC Press, Boca Rayton, Fl, pp.527-616.

3. Formulation of FVIII and liposomes

15 Kogenate (Lot no. 70K027, 620 IU) or New Kogenate (500 IU) was dissolved in 1 ml or 2 ml of H_2O . The rFVIII-SQ concentrate was dissolved in liposome solution. Factor VIII was formulated with liposomes by mixing FVIII concentrate with the liposomes for about 1 hour at room temperature. The ratio of lipids to FVIII units was about 1 mg lipids/1 unit
20 FVIII.

4. Injection into hemophilic mice and bleeding procedures

Factor VIII and FVIII formulated with liposomes were injected into the tail vein of hemophilic mice. The injected dose was 3 units/mouse for
25 Kogenate (2 separate experiments) and New Kogenate and 4 units/mouse for rFVIII-SQ. The mice were bled into citrate tubes at 10 minutes after the injection and at about 4, 19 and 27 hours post-injection.

5. Measurement of FVIII concentrate in mouse plasma

Human FVIII concentrate in mouse plasma was measured using a chromogenic assay (Chromogenix) according to the manufacturer's instructions, and by one stage clotting assay (using Stago reagents and ST4 clotting machine) according to the manufacturer's instructions.

6. Pharmacokinetics analysis

Pharmacokinetics parameters were analyzed using a computer program (RSTRIP, MicroMath Inc.).

10

7. Survival of hemophilic mice following a tail cut

Mice were injected with free rFVIII-SQ or liposome formulated rFVIII-SQ (4 units/mouse, 11 mice in each group. At 20 hours post injection 2 cm of the tail were cut. Tails of the surviving mice were cut again at 28, 44, 52, 69, 88 and 140 hours post-injection (2 mm each time).

Results

The results of FVIII activity at each time point post-injection and the pharmacokinetic parameters [FVIII half-life (HL) and the area under the curve (AUC)] of 4 different experiments are summarized in Tables 4-7B.

20 In Tables 4 and 5, 3 units of Kogenate /mouse were injected; In Table 6, 3 units of New Kogenate /mouse were injected; and in Tables 7A, 7B, and in Fig. 1, 4 units of rFVIII-SQ /mouse were injected.

Table 4: Factor VIII activity (u/ml measured by a chromogenic assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 4.5 hours	T = 19 hours	T = 27 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
Kogenate (n=7)	Average (u/ml) \pm SD	2.878 \pm 0.571	0.450 \pm 0.392	0.015 \pm 0.0043	0.0016 \pm 0.004	7.218	1.619
Injected Material		T = 10 min.	T = 3.6 hours	T = 18.1 hours	T = 26.1 hours		
Kogenate + liposomes (n=7)	Average (u/ml) \pm SD	2.951 \pm 0.333	1.121 \pm 0.337	0.023 \pm 0.003	0.014 \pm 0.0018	10.969	2.460

Table 5: Factor VIII activity (u/ml measured by a chromogenic assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 3.334 hours	T = 19.334 hours	T = 26.3 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
Kogenate (n=8)	Average (u/ml) \pm SD	3.686 \pm 0.674	0.960 \pm 0.469	0.0128 \pm 0.007	0.0025 \pm 0.006	9.314	1.632
Injected Material		T = 10 min.	T = 3.5 hours	T = 19.5 hours	T = 26.5 hours		
Kogenate + liposomes (n=8)	Average (u/ml) \pm SD	3.618 \pm 0.982	1.571 \pm 0.137	0.032 \pm 0.009	0.012 \pm 0.009	15.059	2.771

Table 6: Factor VIII activity (u/ml measured by a chromogenic assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 2.5 hours	T = 17 hours	T = 26 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
New Kogenate (n=4)	Average (u/ml) \pm SD	1.841 \pm 0.643	0.120 \pm 0.036	0.004 \pm 0.003	0.0015 \pm 0.003	1.910	0.592
Injected Material		T = 10 min.	T = 2.666 hours	T = 17.166 hours	T = 26.166 hours		
New Kogenate + liposomes (n=6)	Average (u/ml) \pm SD	2.393 \pm 0.243	0.352 \pm 0.131	0.011 \pm 0.0019	0.008 \pm 0.001	3.544	0.904

Table 7A: Factor VIII activity (u/ml measured by a chromogenic assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 4.166 hours	T= 20.166 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
RVIII (n=10)	Average (u/ml) \pm SD	3.937 \pm 0.449	0.444 \pm 0.131	0	7.9	1.270
Injected Material		T = 10 min.	T = 4.5 hours	T= 20.5 hours		
RVIII + liposome s (n=11)	Average (u/ml) \pm SD	3.828 \pm 1.08	0.555 \pm 0.198	0.005 \pm 0.008	9.249	1.555

Table 7B: Factor VIII activity (u/ml measured by a one stage clotting assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 4.166 hours	T= 20.166 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
RVIII (n=9)	Average (u/ml) \pm SD	4.181 \pm 1.275	1.742 \pm 0.778	0.414 \pm 0.085	21.246	3.521
Injected Material		T = 10 min.	T = 4.5 hours	T= 20.5 hours		
RVIII + liposome s (n=10)	Average (u/ml) \pm SD	3.305 \pm 0.831	2 \pm 0.709	0.531 \pm 0.147	28.718	6.900

5

In addition, the half-life of human FVIII in each mouse was calculated and the FVIII half-lives in all the experimental groups were statistically compared to each other by a student t-test.

10

The statistical analysis indicates that in all 4 experiments human FVIII half-lives in the groups that received liposome-formulated FVIII were higher and significantly different ($p < 0.055$) from human FVIII half-lives in the groups that received free FVIII:

- HL of Kogenate versus HL of Kogenate + liposomes (table 4) $p=0.054$;
HL of Kogenate versus AUC of Kogenate + liposomes (table 5) $p=0.031$;
HL of New Kogenate versus HL of New Kogenate + liposomes (table 6)
 $p=0.0085$;
5 HL of rFVIII-SQ versus HL of rFVIII-SQ + liposomes (table 7A)
 $p=0.0045$; HL of rFVIII-SQ versus HL of rFVIII-SQ + liposomes (table
7B) $p=0.022$).

These results indicate that the formulation of Kogenate, New
Kogenate or rFVIII-SQ with liposomes significantly increases the factor
10 half-life (HL) and the area under the curve (AUC) of FVIII in hemophilic
mice (factor of 1.6-2.0 for HL and AUC).

Survival of the mice is illustrated in Fig. 1. The results of the
tail cut experiment indicate that the liposome formulated FVIII is biologically
active longer than free FVIII, and therefore can protect hemophilic patients
15 for a longer period of time.

Example 5

Effectiveness of FVIII composition in patients with inhibitors

15 units of FVIII (Kogenate) were incubated for one hour at
room temperature with 120 nm liposomes (15 mg lipids) containing
20 EPC:DSPE-PEG2000 (95:5 mole %). Then, 1 unit of free FVIII (Kogenate)
or 1 unit of liposome formulated FVIII were incubated for 2 hours at 37°C
with various dilutions of a serum from a hemophilia patient who had
developed inhibitors (anti FVIII antibodies). After the incubation, the activity
of factor VIII was measured by a chromogenic assay.

25 The results are summarized in table 8:

Table 8: Activity (units/ml) of factor VIII in the presence of FVIII inhibitors

Serum dilution	Free FVIII	FVIII-liposomes
None	0	0.028
1:5	0.052	0.094
1:10	0.137	0.162
1:25	0.6	0.98
1:100	8.736	14.94

- 5 It can clearly be seen from this experiment that administration of FVIII together with the colloidal particles is effective in protecting the FVIII from serum inhibitors.

CLAIMS:

1. A pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, said particles comprising
5 approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,
wherein said FVIII is not encapsulated in said colloidal particles.
- 10 2. The pharmaceutical composition of Claim 1 wherein the colloidal particle has a mean particle diameter of between about 0.05 to about 0.4 microns.
3. The pharmaceutical composition of Claim 2 wherein the colloidal particle has a mean particle diameter of approximately 0.1 microns.
- 15 4. The pharmaceutical composition of Claim 1 wherein said amphipathic lipid is a phospholipid from natural or synthetic sources.
5. The pharmaceutical composition of Claim 4 wherein said amphipathic lipid is egg-phosphatidylcholine.
6. The pharmaceutical composition of Claim 1 wherein said
20 biocompatible hydrophilic polymer is selected from the group consisting of polyalkylether, polylactic and polyglycolic acid families.
7. The pharmaceutical composition of Claim 6 wherein said biocompatible hydrophilic polymer is polyethylene glycol.
8. The pharmaceutical composition of Claim 7 wherein the
25 polyethylene glycol has a molecular weight of between about 1000 to about 5000 daltons.
9. The pharmaceutical composition of Claim 8 wherein the polyethylene glycol has a molecular weight of approximately 2000 daltons.

10. The pharmaceutical composition of Claim 1 wherein the FVIII is from a natural source.
11. The pharmaceutical composition of Claim 1 wherein the FVIII is recombinantly prepared.
- 5 12. The pharmaceutical composition of Claim 1 wherein the particle to FVIII ratio (w/unit FVIII) is between about 0.1 mg/unit and about 10 mg/unit.
13. The pharmaceutical composition of Claim 12 wherein the particle to FVIII ratio (w/unit FVIII) is approximately 1 mg/unit.
- 10 14. Method of treatment of a patient suffering from hemophilia comprising administering to said patient a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic
- 15 lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,
- wherein said FVIII is not encapsulated in said colloidal particles.
15. A method according to Claim 14 wherein said patient has
- 20 developed FVIII inhibitor antibodies.
16. Use of a colloidal particle in the preparation of a pharmaceutical composition for parenteral administration for treatment of a patient suffering from hemophilia comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral
- 25 colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,
- wherein said FVIII is not encapsulated in said colloidal particles.

17. A use according to Claim 15 wherein said patient has developed FVIII inhibitor antibodies.

18. A pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of a protein or polypeptide and substantially neutral colloidal particles, said particles comprising
5 approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,

wherein said protein or polypeptide is selected from the group
10 consisting of:

(a) proteins or polypeptides capable of externally binding said colloidal particles; and

(b) proteins or polypeptides capable of binding polyethylene glycol,

and wherein said protein or polypeptide is not encapsulated in
15 said colloidal particles.

19. Use of a colloidal particle in the preparation of a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of a protein or polypeptide and substantially neutral colloidal particles, said particles comprising
20 approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,

wherein said protein or polypeptide is selected from the group consisting of:

25 (a) proteins or polypeptides capable of externally binding said colloidal particles; and

(b) proteins or polypeptides capable of binding polyethylene glycol,

and wherein said protein or polypeptide is not encapsulated in said colloidal particles.

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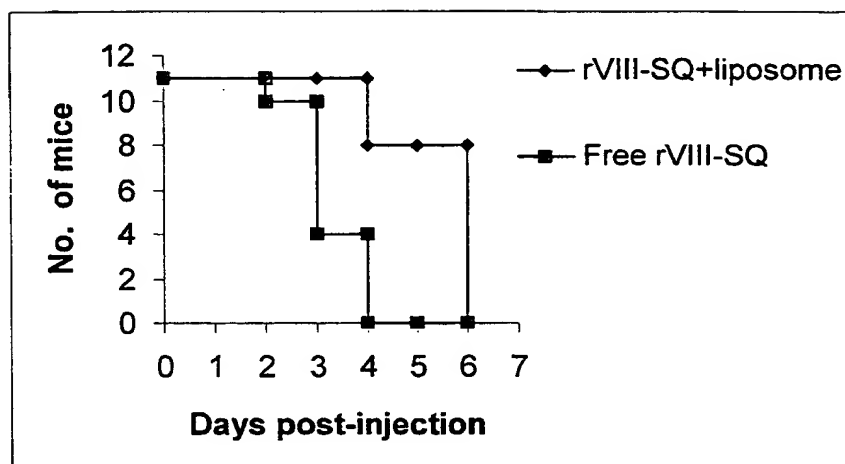


Fig. 1

INTERNATIONAL SEARCH REPORT

Int: lional Application No

PCT/IL 99/00217

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127 A61K38/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 013 556 A (WOODLE MARTIN C ET AL) 7 May 1991 (1991-05-07) cited in the application	19
Y	column 12, line 7-21 abstract	1-18
Y	SAHLI H ET AL: "Interactions of poly(lactic acid) and poly(lactic acid-co-ethylene oxide) nanoparticles with the plasma factors of the coagulation system" BIOMATERIALS, vol. 18, no. 4, 1 February 1997 (1997-02-01), page 281-288 XP004017219 ISSN: 0142-9612 abstract	1-18

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

29 July 1999

Date of mailing of the international search report

05/08/1999

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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 21235 A (LIPOSOME TECHNOLOGY INC) 29 September 1994 (1994-09-29) cited in the application the whole document ---	1-19
A	G. BLUME ET AL: "Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1149, 1993, pages 180-184, XP000406105 Amsterdam, NL abstract -----	1-19

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/IL 99/00217

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5013556 A	07-05-1991	AT 115401 T	15-12-1994
		AT 122229 T	15-05-1995
		AU 642679 B	28-10-1993
		AU 6637490 A	16-05-1991
		AU 654120 B	27-10-1994
		AU 6898291 A	16-05-1991
		CA 2067133 A,C	21-04-1991
		CA 2067178 A,C	21-04-1991
		DE 69015207 D	26-01-1995
		DE 69015207 T	04-05-1995
		DE 69019366 D	14-06-1995
		DE 69019366 T	05-10-1995
		DK 496835 T	17-07-1995
		EP 0496813 A	05-08-1992
		EP 0496835 A	05-08-1992
		ES 2071976 T	01-07-1995
		FI 921763 A	21-04-1992
		FI 921764 A	21-04-1992
		GR 3017060 T	30-11-1995
		HK 14097 A	14-02-1997
		IL 96069 A	08-12-1995
		IL 96070 A	30-03-1995
		JP 10001431 A	06-01-1998
		JP 2667051 B	22-10-1997
		JP 5501264 T	11-03-1993
		JP 5505173 T	05-08-1993
		KR 134982 B	22-04-1998
		LU 88854 A	11-03-1997
		NO 304637 B	25-01-1999
		US 5527528 A	18-06-1996
		WO 9105545 A	02-05-1991
		WO 9105546 A	02-05-1991
		US 5620689 A	15-04-1997
		US 5225212 A	06-07-1993
		US 5213804 A	25-05-1993
		US 5843473 A	01-12-1998
		US 5356633 A	18-10-1994
WO 9421235 A	29-09-1994	AT 175868 T	15-02-1999
		AU 6368494 A	11-10-1994
		CA 2157410 A	29-09-1994
		DE 69416116 D	04-03-1999
		DE 69416116 T	24-06-1999
		EP 0689428 A	03-01-1996
		JP 8508256 T	03-09-1996

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